Polyoxyethylene 40 Stearate Modulates Multidrug Resistance and Enhances Antitumor Activity of Vinblastine Sulfate

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Abstract

Multidrug resistance (MDR) is one of the major obstacles limiting the efficacy of cancer chemotherapy. Identification of new and effective MDR reversal agents is needed. In this study, the effects of polyoxyethylene 40 stearate (PS40) on MDR were evaluated via the transport of the P-glycoprotein (P-gp) substrate vinblastine sulfate (VBL) through Caco-2 cell monolayers and rat intestine tissue. The effects of PS40 on the antitumor activity of VBL were examined through 3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay and multidrug-resistant tumor-bearing mice. Results of the transport experiments showed that PS40 reduced VBL efflux. The cytotoxicity of vinblastine to K562/ADR cells was significantly enhanced when the cells were cotreated with 100 or 150 μg/mL PS40. In vivo data revealed that average tumor volume and average tumor weight were significantly less in the VBL+PS40 group than in the VBL group. The inhibition rate for tumor growth was increased from 0.06 (VBL group) to 0.84 (VBL+PS40 group). These results suggest that PS40 may be a potentially useful adjuvant to enhance the therapeutic effects of P-gp substrates.

Keywords: Polyoxyethylene 40 stearate, P-glycoprotein, vinblastine sulfate, Caco-2, nude mice, K562/ADR

Introduction

A major limitation to the successful chemotherapeutic treatment of cancer is the resistance of tumor cells to cytotoxic drugs. This phenomenon is called multidrug resistance (MDR). In humans, several transporters have been identified that can cause resistance in tumor cells: MDR1 P-glycoprotein (P-gp) and the multidrug resistance proteins (MRPs).[1-5] These proteins actively transport drugs out of the cell, resulting in a decreased intracellular drug concentration. The overexpression of drug pumps in tumor cells has led to a search for compounds that can inhibit these transporters in clinical therapy. These compounds should preferably (1) be selective for 1 or more transporters and have a high affinity to bind to the transporters, (2) be nontoxic, and (3) be stable in human blood.[6] Although several P-gp inhibitors have been developed,[7-12] none are currently used in the clinical environment because of unwanted toxic side effects at concentrations necessary for efficacy. For MDR inhibition to be successful, low-toxicity pharmaceuticals or agents need to be used.

Polyoxyethylene 40 stearate (PS40) is a non-ionic surfactant. Non-ionic surfactants can be used as solubilizers, emulsifiers, and oral absorption enhancers for improving the bioavailability of poorly absorbed drugs. In several studies, PS40 was found to improve the absorption of P-gp substrates such as cyclosporine A and epirubicin.[13,14] However, PS40's potential for reversing MDR in cancer chemotherapy has not been thoroughly assessed. Therefore, the purpose of this study was to evaluate the effects of PS40 on MDR in vitro and in vivo. In the study, vinblastine sulfate (VBL), which is clinically used in the treatment of many kinds of cancers,[15] was used to examine the effects of PS40 on MDR. Transport experiments through human colon adenocarcinoma (Caco-2) cell monolayers and rat intestine tissue were performed to determine whether PS40 could modulate MDR in vitro. Using the doxorubicin-resistant human chronic myelogenous leukemia cell line K562/ADR, we determined the reversal effects of PS40 on the resistance to VBL by means of a cytotoxicity assay with 3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Multidrug-resistant tumor-bearing mice experiments were performed to investigate whether PS40 could enhance the cancer therapy effects of VBL.

Materials and Methods

Materials

The Caco-2 cell line was purchased from the American Type Culture Collection through Beijing Zhongyuan Tech Ltd (Beijing, China). The doxorubicin-resistant human chronic myelogenous leukemia cells (K562/ADR) were kindly provided by Professor Xu Qiang (State Key Laboratory of Pharmaceutical Biotechnology, Nanjing University, Nanjing, China). Modified minimum essential medium with L-glutamine and without sodium bicarbonate, as well as fetal bovine serum, were purchased from Hyclone Co through Genetimes Technology Inc (Shanghai, China). Bovine

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serum was purchased from Lanzhou National Hyclone Bio-Engineering Co Ltd (Lanzhou, China). VBL was purchased from Nantong Diligent Pharmaceutical Factory (Jiangsu, China). PS40 was purchased from Nanjing WELL Chemical Corporation, Ltd (Nanjing, China). Sprague-Dawley rats were purchased from the Jiangning Qinglongshan animal cultivation farm (Nanjing, China). BALB/c-nu/nu mice were purchased from the Beijing Laboratory Animal Research Center (Beijing, China).

Cell Culture

Caco-2 cells were cultured as described previously.[16] Briefly, cells were cultured at 37°C in the modified minimum essential medium with L-glutamine and without sodium bicarbonite, supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin in an atmosphere of 5% CO_2 and 100% humidity. The cells were passaged upon reaching ~80% to 90% confluency and plated at a density of 80 000 cells/mL in T flasks. Caco-2 cells (passage number 45-50) were seeded at a density of 80 000 cells/mL on polycarbonate membranes of Transwells (PI1250, Millipore, Billerica, MA). The medium, added to the apical (AP) and basolateral (BL) compartments, was changed the day after seeding and every other day thereafter. The cell monolayers were used ~21 days after seeding.

K562/ADR cells were cultured at 37°C in RPMI1640, supplemented with 10% bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin in an atmosphere of 5% CO_2 and 100% humidity. The cells were passaged every other day and plated at a density of 80 000 cells/mL in T flasks.

Transport Experiments in Caco-2 Cell Monolayers

Caco-2 cell monolayers were used when the transendothelial electrical resistance (TEER) exceeded 300Ω×cm^2. The transport studies were performed as described in previous reports.[14,16] The cells were rinsed twice with transport buffer solution (TBS, Hanks balanced salt solution with 25 mM HEPES, pH 7.4) and then incubated at 37°C (temperature maintained throughout the experiment) in 1 mL of TBS for 30 minutes. Donor solutions of 200 μg/mL VBL with or without different concentrations of PS40 in TBS were added to the donor compartment. The final concentrations of PS40 were 50, 100, and 150 μg/mL. The AP compartment and the BL compartment served as the donor compartments for absorptive (AP-BL) and efflux (BL-AP) transport, respectively. Blank TBS was added to the acceptor compartment. At 20, 40, 60, 90, 120, and 150 minutes, aliquots of 100 μL were withdrawn from the acceptor side for analysis and immediately replaced by fresh TBS medium. Sink conditions and a constant temperature were maintained throughout the transport experiments. TEER was measured to check whether the cell monolayers' integrity and viability had been adversely affected under the experimental conditions. All transport studies were performed at 37°C and 50 oscillations/min. Each experiment was repeated 3 times (n = 3).

Transport Experiments in Rat Intestine Tissue

All procedures were performed in accordance with the guidelines and approval of the local Institutional Animal Experimentation Ethics Committee. Mature female Sprague-Dawley rats were fed with standard laboratory food. The transport experiments performed were similar to those described previously.[17] After a 24-hour fast, rats were sacrificed by decapitation and ~20 cm long segments of intestine were removed. The intestine tissues were immediately rinsed with ice-cold Krebs-Ringer medium (in mM: 151.5 Na^+, 4.7 K^+, 2.3 Mg^{2+}, 3.3 Ca^{2+}, 149.3 Cl^-, 1.8 $H_2PO_{4-}^-$, 16.3 HCO_3^-, 7.7 glucose) to remove the luminal content. The unstripped intestine tissues were cut into pieces of 3 to 5 cm in length, opened along the mesenteric border, and then carefully segregated. The muscular layer was scraped to obtain the mucosal layer of the intestine.

A diffusion apparatus (transdermal diffusion apparatus TP-3, Nanjing Xinlian Electronic Equipment Co, Ltd, Nanjing, China) was used in the experiments. Pieces of intestinal tissue were placed as flat sheets between the donor chamber and the acceptor chamber. Donor solutions of 200 μg/mL VBL without or with 50, 100, and 150 μg/mL of PS40 in Krebs-Ringer medium were added separately to initiate the experiment. For absorptive experiments, the mucosal side was exposed to the donor solution, while for efflux (BL-AP) transport, the mucosal side was exposed to the acceptor solution. Blank Krebs-Ringer medium was added to the acceptor chambers. The solution in the donor chamber and the acceptor chamber was bubbled with 95% O_2/5% CO_2 and maintained at 37°C throughout the experiments. Magnetic stirrers working at 150 rpm were placed in the acceptor chamber throughout the experiment. At 30-minute intervals during the 180-minute study, aliquots of 400 μL were withdrawn from the acceptor chamber for analysis and immediately replaced by fresh Krebs-Ringer medium. Sink conditions were maintained throughout the transport experiments. Each experiment was repeated 3 times (n = 3).

Cytotoxicity Assay

The effects of PS40 on VBL's cytotoxicity to K562/ADR cells were determined in quadruplicate using MTT cell viability assay as previously described.[18] PS40 was added to the test VBL solution. The cytotoxicity of VBL to K562/ADR cells was then assessed. The final concentrations of PS40 were 0, 50, 100, and 150 μg/mL, while the final concentrations of VBL were 50, 100, 120, 180, 200, and 220

μg/mL. After 8 hours of treatment, cells were incubated for 4 hours in the presence of MTT reagent and then lysed with dimethyl sulfoxide. Absorbance was measured at $A_{490\ nm}$. Each experiment was repeated at least 4 times ($n \geq 4$).

In Vivo Study Using Multidrug-Resistant Tumor-Bearing Nude Mice Models

All procedures were performed in accordance with the guidelines and approval of the local Institutional Animal Experimentation Ethics Committee. Female BALB/c-nu/nu mice were age-matched (4 weeks of age) at the beginning of each experiment. All mice were maintained in specific pathogen-free facilities and fed with irradiated food. The K562/ADR cells were inoculated subcutaneously into the left side of the armpit of athymic mice (1×10^7 cells/each). The mice were assigned randomly to 3 groups after inoculation (n = 4 in each group).

After tumors reached 2.0 mm × 2.0 mm, mice in the control group were treated with 0.9% sodium chloride solution. VBL was dissolved in the 0.9% sodium chloride solution, yielding a concentration of 200 μg/mL. The drug was injected subcutaneously in a volume of 0.1 mL per 10 g of body weight at a dosage of 2 mg/kg VBL around the tumor every other day for 8 days (VBL group). VBL and PS40 were dissolved in 0.9% sodium chloride solution, yielding a solution of 200 μg/mL VBL and 150 μg/mL PS40. The drug was injected subcutaneously in a volume of 0.1 mL per 10 g of body weight at a dosage of 2 mg/kg VBL and 1.5 mg/kg PS40 around the tumor every other day for 8 days (VBL+PS40 group). The volume of tumors and the weight of mice were measured every day from the day the tumors were formed. Tumor dimensions were measured with digital calipers to obtain 2 diameters. Tumor size was calculated using the following formula[19]: tumor volume = $\pi/6 \times a \times b^2$, where a is the longer diameter and b is the shorter diameter.

The inhibition rate was calculated using the following formula: inhibition rate = $1 - W_t/W_c$, where W_t is the average tumor weight in the test group and W_c is the average tumor weight in the control group.

Sample Analysis and Statistical Analysis

VBL stock solutions were prepared in water in the range of 0.02 to 2 mg/mL and stored at 4°C. For daily calibration, each stock solution was diluted 1:50 with the mobile phase and calibration curves were recorded. VBL samples were centrifuged for 2 minutes at 4°C and 10 000 rpm, each supernatant was transferred into a new tube, and 20-μL aliquots were injected for analysis by high-performance liquid chromatography. The assay conditions were as follows: samples were subjected to a 300 × 4 mm C18 YWG column, eluted with an isocratic mobile phase consisting of methanol:water:acetic acid:triethylamine = 62:28:0.3:0.3 at a flow rate of 1 mL/min, and detected at 264 nm.

The apparent permeation coefficient (P_{app}) was calculated using the following formula[20]: $P_{app} = (dQ/dt)/(A \times C_0)$, where Q is the accumulated drug permeation, A is the permeation area, and C_0 is the initial concentration. Because there was a lag period before transport became stable, the initial time point (t = 0) was not used for the calculation.

Data were presented as mean ± SD. The statistical significance of means for the studies was determined by analysis of variance followed by the Dunnett post hoc test. *P* values for significance were set at .05.

Results and Discussion

Transport Experiment in Caco-2 Cell Monolayers and in Rat Intestine Tissue

Throughout the experiments, TEER was not reduced. The transport of VBL across Caco-2 cell monolayers was linear with respect to time. The P_{app} values are presented in Figure 1. Compared with the control, the P_{app} values for absorption (AP-BL) were significantly higher with 150 μg/mL PS40, and the P_{app} values for efflux (BL-AP) were significantly lower with 100 and 150 μg/mL PS40 ($P < .05$).

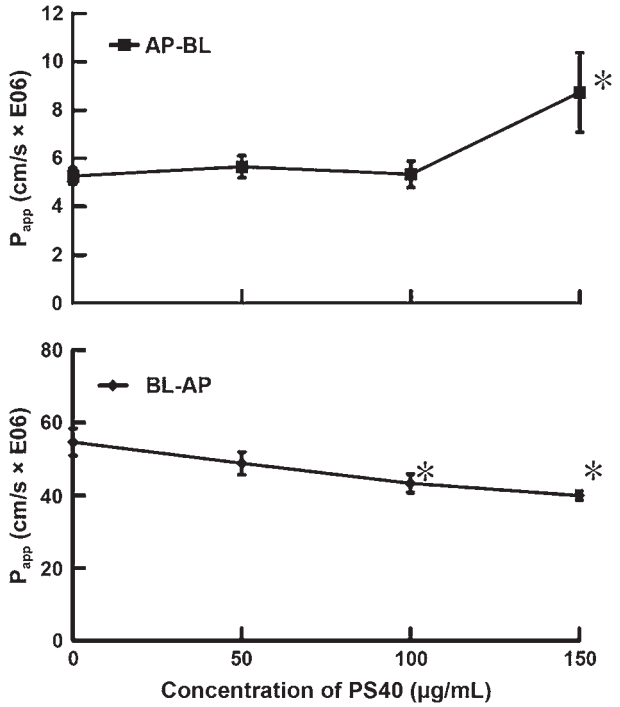

Figure 1. P_{app} (cm/s × E06) values of vinblastine sulfate for transport experiments performed using Caco-2 monolayers with or without different concentrations of PS40 (mean ± SD, n = 3). *$P < .05$ vs control value. AP indicates apical; BL, basolateral; PS40, polyoxyethylene 40 stearate.

Generally, PS40 reduced the BL-AP to AP-BL ratio (ie, the B/A ratio) of VBL transport. In the absence of PS40, the B/A ratio was 10. With 50, 100, and 150 μg/mL PS40, the ratios were 9, 8, and 4, respectively.

The transport of VBL across the rat intestine tissue was linear with respect to time, and the P_{app} values are presented in Figure 2. Compared with the control, the P_{app} values for efflux (BL-AP) were significantly lower with 100 μg/mL ($P < .05$) and 150 μg/mL ($P < .01$) PS40. Generally, PS40 reduced the B/A ratio of VBL transport. In the absence of PS40, the B/A ratio was 1.80. With 50, 100, and 150 μg/mL PS40, the ratios were 1.50, 0.88, and 0.58, respectively.

As shown in Figures 1 and 2, the P_{app} values for the control group ($C_{PS40} = 0$) in Caco-2 cell monolayers differed from those in rat intestine tissue. Thus, the P_{app} value for BL-to-AP transport in rat intestine tissue was ~2 times that in Caco-2 cell monolayers, while for AP-to-BL transport, the ratio was ~15. The discrepancy may be due to the differences between Caco-2 cell monolayers and rat intestine. Caco-2 cells are derived from a human colonic tumor and exhibit permeability characteristics different from those of normal epithelium.[21] Compared with in the rat intestine membrane, in the Caco-2 monolayer, resistance is high and permeability is low. Furthermore, P-gp expression is higher in Caco-2 than in rat intestine. Studies using Caco-2 cell monolayers have shown that transepithelial permeabilities of classic P-gp substrates such as vinblastine are modified by active transport via P-gp.[22,23] Despite these differences, the effects of PS40 on VBL transport in the Caco-2 monolayer and in rat intestine tissue were similar. PS40 enhanced apparent AP-BL transport at 150 μg/mL in rat intestine tissue and decreased drug efflux at 100 and 150 μg/mL in Caco-2 cell monolayers and in rat intestine tissue.

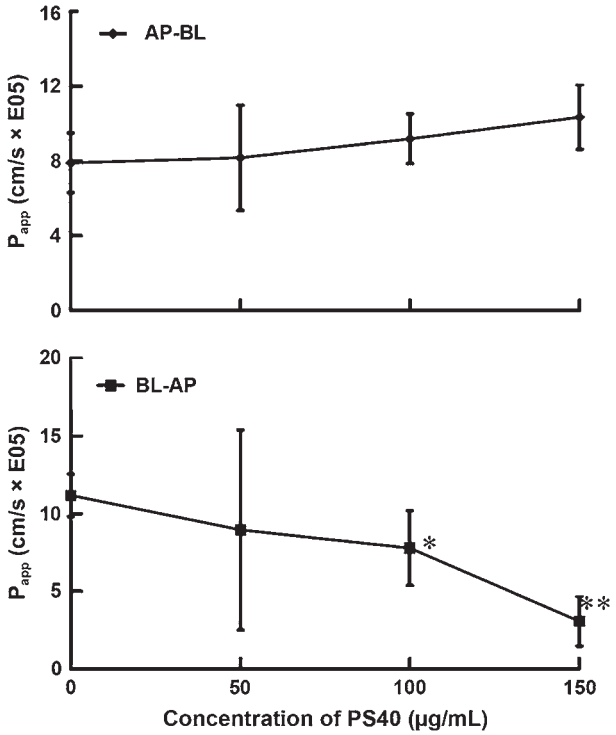

Figure 2. P_{app} (cm/s × E05) values of vinblastine sulfate for transport experiments performed using rat intestine tissues with or without different concentrations of PS40 (mean ± SD, n ≥ 4). *$P < .05$, **$P < .01$ vs control value. AP indicates apical; BL, basolateral; PS40, polyoxyethylene 40 stearate.

In the gut, P-gp and MRP2 are localized in the apical membrane of enterocytes. By inducing a net BL-to-AP flux of xenobiotics, P-gp and MRP2 can act as a barrier to the intestinal absorption of drugs.[24-27] P-gp and MRP2 are also known to be located in the AP (brush-border) membrane of Caco-2 cells. Vinblastine is known to be effluxed by P-gp and MRP2. We thus speculated that the function of P-gp, MRP2, and other transporters responsible for the efflux of vinblastine in the intestines might be influenced by the presence of PS40.

For the modulation of MDR by nonionic surfactants, competitive inhibition of substrate binding, alteration of membrane fluidity, and inhibition of efflux pump ATPase have been proposed as possible mechanisms. Xia and Onyuksel found that surfactant monomers can inhibit efflux transporters by membrane perturbation, as reported previously for P-gp.[28] Bogman et al indicated that surfactants inhibited P-gp by transporter-specific interaction rather than nonspecific membrane perturbation.[29] Collnot et al regarded ATPase inhibition as an essential factor in the inhibitory mechanism of tocopheryl polyethylene glycol 1000 succinate on cellular efflux pumps.[30] Seelig and Gerebtzoff reviewed the membrane binding propensity of different noncharged detergents and discussed their ability to bind to P-gp.[31] Most of these active surfactants contain similar structural elements, such as polyethylene oxide groups and medium fatty acid chain length. Lo indicated the relationships between the hydrophile-lipophile balance values of several surfactants and their MDR modulating effect.[14] PS40 contains these structural elements and modulated MDR in the study. Further studies are needed to determine the mechanisms involved in the MDR-modulating effect mediated by PS40.

Cytotoxicity Assay and Drug Efficacy Study in Multidrug-Resistant Tumor-Bearing Nude Mice Models

K562/ADR cell survival rates after treatment with different concentrations of PS40 and VBL are summarized in Figure 3. When the concentration of VBL was increased from 0 to 220 μg/mL, the survival rates of K562/ADR cells decreased. Compared with the control ($C_{PS40} = 0$), cell survival rates at the same concentration of VBL were significantly lower with 100 and 150 μg/mL PS40, especially when the concentration of VBL was relatively low.

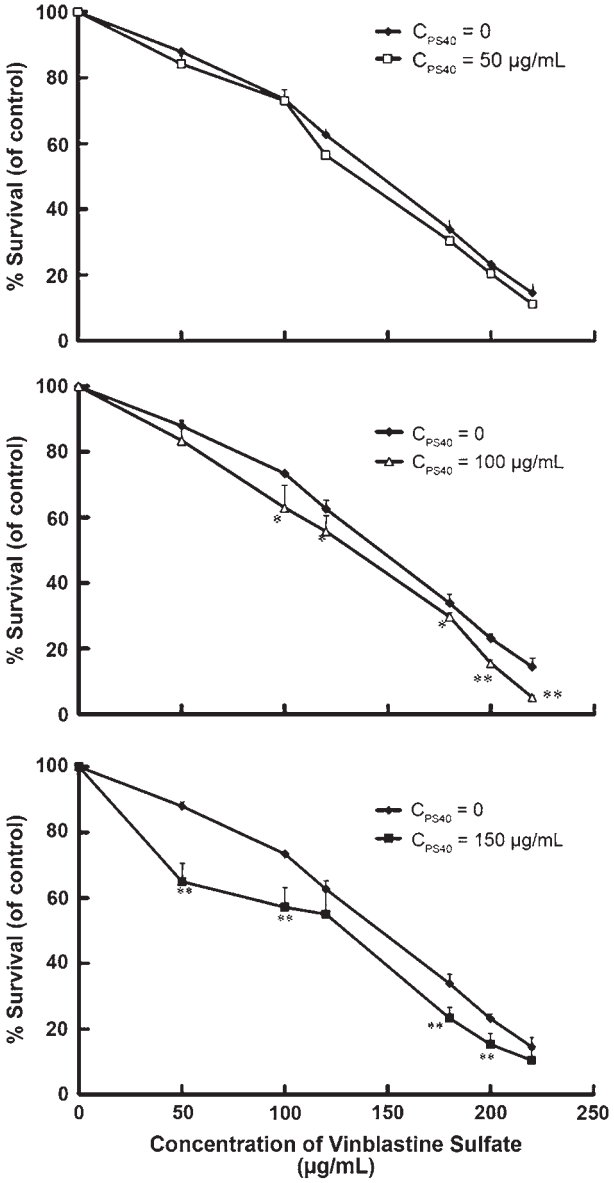

Figure 3. K562/ADR cell survival rates after treatment with PS40 and vinblastine sulfate at different concentrations (mean ± SD, n ≥ 4). **P* < .05, ***P* < .01 vs control value. PS40 indicates polyoxyethylene 40 stearate.

Xenograft tumors were formed between 10 and 20 days after K562/ADR cells were inoculated into mice. The tumor volume (mm^3) and the tumor weight (g) for the 3 groups are shown in Figure 4. There was no significant difference in the mean volume of tumors between the control group and the VBL group: they were 431 ± 104 mm^3 and 322 ± 100 mm^3, respectively. Meanwhile, the mean volume of tumors in the VBL+PS40 group was significantly lower ($P < .05$): 137 ± 45 mm^3. Similarly, there was no significant difference in the mean tumor weight between the control group and the VBL group, while the mean tumor weight in the VBL+PS40 group was significantly lower ($P < .01$). The inhibition rates of tumor growth in the VBL group and the VBL+PS40 group were 0.06 and 0.84, respectively.

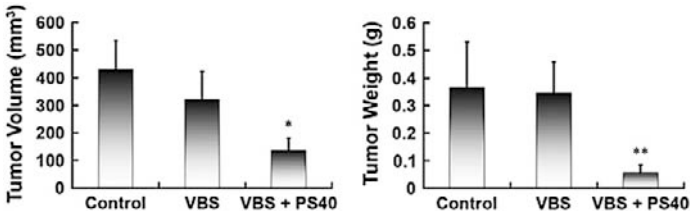

Figure 4. Tumor volume and tumor weight for the 3 groups in the drug efficacy study in multidrug-resistant tumor-bearing mice models (mean ± SD, n = 4). **P* < .05, ***P* < .01 vs control value. VBS indicates vinblastine sulfate; PS40, polyoxyethylene 40 stearate.

PS40 is a non-ionic surfactant that is widely used in commercial dosage forms to solubilize drugs.[32] PS40's toxicity and safety have been studied. There are no data suggesting that PS40 alone can inhibit tumor growth. By oral administration, the median lethal dose of PS40 for rat is 32 000 mg/kg.[33] In short-term and long-term studies with mouse, rat, hamster, dog, monkey, and human,[17,34-39] PS40 produced little or no ocular or dermal irritation and had extremely low acute and chronic toxicities. PS40 is also of little or no reproductive and developmental toxicity, genotoxicity, and carcinogenicity. The most frequent toxicity responses reported include diarrhea and a slight decrease in average body weight. Toxicity data from injection administration are limited. The toxic response produced by PS40 after intravenous injection to dogs was prolonged hypotension.[33] PS40 could not increase the in vitro permeability of Yoshida ascites cells to eosin Y.[40] Overall, PS40 is of low or negligible toxicity, and it is reasonable to infer that PS40 alone could not inhibit tumor growth. In our study, VBL did not inhibit the growth of multidrug-resistant tumors. But when VBL was cotreated with PS40, the tumor growth was inhibited. Since PS40 alone may not inhibit tumor growth and VBL alone did not inhibit tumor growth, it is reasonable to infer that it is the interaction between PS40 and VBL that accounted for the difference, possibly through inhibition of P-gp by PS40 in vivo.

The cytotoxicity assay and drug efficacy study in tumor-bearing nude mice confirmed that the alleviation/reversion of MDR by PS40 was effective. The result is different from that of Watanabe et al,[41] who found that Cremophor EL reversed MDR in vitro but not in tumor-bearing mouse models. The varying results may be due to the difference in administration routes and dosages but also may be due to differences between Cremophor EL and PS40.

Conclusion

In conclusion, results of this study suggest that PS40 may have MDR-reversing effects. The combined use of VBL and PS40 may enhance VBL's therapeutic efficacy by circumventing drug resistance in cancer.

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